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2019-11

Hui , N , Parajuli , A , Puhakka , R , Grönroos , M , Roslund , M , Vari , H , Selonen , V A O , Yan , G , Siter , N , Nurminen , N , Oikarinen , S , Laitinen , O , Rajaniemi , J , Hyöty , H & Sinkkonen , A 2019 , ' Temporal variation in indoor transfer of dirt-associated environmental bacteria in agricultural and urban areas ' , Environment International , vol. 132 , 105069 . https://doi.org/10.1016/j.env

http://hdl.handle.net/10138/308566
https://doi.org/10.1016/j.envint.2019.105069

Environment International 132 (2019) 105069

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Temporal variation in indoor transfer of dirt-associated environmental bacteria in agricultural and urban areas

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ARTICLE INFO

Handling Editor: Yanzheng Gao

Keywords:
Environmental microbiota
Indoor microbiota
Temporal variation
Vegetation

ABSTRACT

An agricultural environment and exposure to diverse environmental microbiota has been suggested to confer protection against immune-mediated disorders. As an agricultural environment may have a protective role, it is crucial to determine whether the limiting factors in the transfer of environmental microbiota indoors are the same in the agricultural and urban environments. We explored how sampling month, garden diversity and animal ownership affected the indoor-transfer of environmental microbial community. We collected litter from standardized doormats used for 2 weeks in June and August 2015 and February 2016 and identified bacterial phylotypes using 16S rRNA Illumina MiSeq sequencing. In February, the diversity and richness of the whole bacterial community and the relative abundance of environment-associated taxa were reduced, whereas human-associated taxa and genera containing opportunistic pathogens were enriched in the doormats. In summer, the relative abundances of several taxa associated previously with beneficial health effects were higher, particularly in agricultural areas. Surprisingly, the importance of vegetation on doormat microbiota was more observable in February, which may have resulted from snow cover that prevented contact with microbes in soil. Animal ownership increased the prevalence of genera *Bacteroides* and *Acinetobacter* in rural doormats. These findings underline the roles of season, living environment and lifestyle in the temporal variations in the environmental microbial community carried indoors. As reduced contact with diverse microbiota is a potential reason for immune system dysfunction, the results may have important implications in the etiology of immune-mediated, non-communicable diseases.

1. Introduction

Microbial exposure affects human health in different ways. Direct pathogen exposure can cause infections, whereas exposure to diverse environmental microbiota likely modulates the human immune system (Strachan, 1989; Rook, 2009; Rintala et al., 2012). The latter is supported by several studies that demonstrate the role of microbial contacts in the development of a normal immune system and protection against immune-mediated non-communicable diseases (e.g. Bach, 2002; Graham-Rowe, 2011). The importance of the interaction with environmental microbiota for the development of a healthy immune system has been recognized by the "biodiversity hypothesis" (Hanski et al., 2012; Haahtela et al., 2015). Modern-day people spend the majority of their time indoors, which limits their exposure to nature and to environmental microbes. Bioaerosol transferred inside through open doors, windows and non-filtered ventilation and household dusts collected from floors, walls and filters of air conditioners have been investigated to understand microbial exposure inside houses (e.g. Dunn et al., 2013; Barberán et al., 2015; Adams et al., 2015; Brągoszewska and Biedroń, 2018; Brągoszewska and Pastuszka, 2018).

Abbreviations: OTU, operational taxonomic unit; rRNA, ribosomal ribonucleic acid; NMDS, non-metric multidimensional scaling; GLMM, generalized linear mixed model

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https://doi.org/10.1016/j.envint.2019.105069
Received 29 March 2019; Received in revised form 28 July 2019; Accepted 29 July 2019

These studies have identified several factors that are associated with the indoor microbial community and they have been used to link indoor exposure to the incidence of immune and other systemic health disorders (Ross et al., 2000; Pakarinen et al., 2008; Dannemiller et al., 2016). Analysis of household dust for the estimation of microbial exposure indoors is particularly relevant in cooler climates, where transfer of environmental microbial communities occurs more easily through shoes and clothing, compared to aerosol transfer from windows and doors, which are mostly kept closed. However, sampling of household dusts poorly differentiates various sources of microbial communities inside the houses (e.g. commensal, household waste and building's microbiota) from those imported from outdoors. Therefore, the study of outdoor environmental factors on the indoor microbial environment and factors affecting the transfer can provide valuable insights into the indoor exposure to environmental microbiota.

The indoor-transfer of environmental microbiota is likely affected by the meteorological conditions including temperature, humidity, wind speed and direction, and illumination, living environment including nearby land cover (Parajuli et al., 2018; Roslund et al., 2019) and lifestyle of the residents (Raisi et al., 2010; Brągoszewska and Pastuszka, 2018). Owing to these facts, we used experimental doormats inside Finnish houses in our recent study to examine the role of outdoor land cover type on the transfer of microbial community indoors (Parajuli et al., 2018). However, we did not study the role of garden plant diversity in the immediate vicinity of residential area on the indoor-transfer of outdoor microbiota. Plants harbour diverse microbial communities (including epiphytes and endophytes) and also influence the composition of soil microbiota, particularly in the rhizosphere and phyllosphere (Berg and Smalla, 2009; Berg et al., 2014; Płociniczak et al., 2016; Pacwa-Płociniczak et al., 2018). Therefore, garden diversity plausibly influences human exposure to environmental and particularly plant-associated microbes, which may have implications on immune functioning and overall health in humans. However, studies on the role of outdoor plants in shaping the indoor microbiota are lacking. Indoor microbiota are believed to be contributed indirectly by outdoor plants via air flow or directly by indoor plants (Burge et al., 1982; Berg et al., 2014). To our knowledge, there have been no attempts to study the role of garden plant diversity on indoor transfer of environmental microbiota.

Another natural phenomenon that may influence microbial exposure among humans is the season, which is characterized by temperature, humidity, sunlight etc. and likely affect the microbial composition of the environment. Seasonal patterns of many infectious and immune-mediated diseases of public health importance are recognized. However, the mechanisms underlying the seasonality of human-microbe interactions are not well understood, partly because previous studies have concentrated on airborne and indoor dust microbiota (Altizer et al., 2006; Fisman, 2007; Monsalvo et al., 2011) and largely omitted microbial transfer through litter indoors (Parajuli et al., 2018). The incidences of some common diseases follow a seasonal pattern and therefore understanding how season affects the temporal dynamics of the indoor microbial community could provide insight into the epidemiology of seasonal infectious diseases (Grassly and Fraser, 2006). Previous studies have revealed temporal variations on microbial communities in indoor and outdoor environments (Rintala et al., 2008; Franzetti et al., 2011; Frankel et al., 2012). Low temperatures are often associated with lower overall microbial abundance than in warmer seasons (Yergeau et al., 2010; Bertolini et al., 2013). Possibly because humans spend more time indoors when the weather is cold and rainy, the microbes that originate from household residents are more abundant in the indoor dust in winter than in summer (Rintala et al., 2012). However, those results are from indoor dust studies and the extent of temporal variation in the indoor transfer of dirt-attached environmental microbial community has not been studied. As microbial communities in dirt are among the most diverse in the world, improved understanding of this phenomenon will enhance our knowledge on how to minimize the negative consequences of temporal variation, particularly with regards to human health.

In the current study, we placed polyethylene doormats inside the main door of rural and urban houses for 2 weeks in June and August of 2015 and in February 2016 in the Päijät-Häme region of southern Finland and sampled the doormat debris to identify the bacterial communities. The doormat debris was a complex mixture of dirt and minor components such as airborne dust, plant fragments and animal and human scraps; this is therefore an important habitat and suitable sample to study the microbes transferred from the surrounding environment (Parajuli et al., 2018). We used high-throughput sequencing and multivariate statistical analyses to explore how significant is the temporal variation in environmental microbes brought indoors and whether these microbes correlate with plant diversity in the gardens of study volunteers and are affected by animal ownership. We hypothesized that i) the diversity, community structure and relative abundance of environmental bacteria transferred indoors vary between summer months (June and August) and winter (February); ii) the bacterial community in the doormats is influenced by the plant diversity in the gardens of study volunteers; and iii) the abundance of potentially pathogenic bacteria exhibit differential abundance between the summer and winter months.

2. Materials and methods

2.1. Study area and participants

The participants in our study were 61 elderly retired people aged 65 to 79 years (see Parajuli et al., 2018). Thirty participants lived in detached houses in rural agricultural areas (in the region of Päijät-Häme and the municipalities of Iitti and Pukkila) and 31 resided in apartments in urban areas (in the city of Lahti) in Southern Finland. The characteristics of study participants and study sites, including the criteria for rural-urban classification are described in more detail in our recent study (Parajuli et al., 2018) and presented in Table S1. Out of the 30 rural participants, 14 owned pet(s) or other domestic animals (10 households with cats, 5 with dogs, 3 with chickens, 2 with cows, 1 with horses and 1 with pigs). Because only 3/34 participants from the urban areas owned an animal, we decided to exclude them from further analyses.

This study was carried out according to the recommendations of the "*Finnish Advisory Board on Research Integrity*" and an ethical statement was obtained from the local ethics committee (*Tampereen yliopistollisen sairaalan erityisvastuualueen alueellinen eettinen toimikunta*). Written informed consent was obtained from all subjects in accordance with the *Declaration of Helsinki*.

2.2. Doormat installation and material recovery

New, unused, scraper-type polyethylene doormats of size 45 × 57 cm were placed at the main entrance door of the study participants at two different time points in the year 2015 (June and August) and once in 2016 (February) for a period of 2 weeks. Participants were instructed properly to wipe their feet when entering their house and not to clean the doormat during the study period. Materials deposited in the doormats were collected at the end of study period as described in Parajuli et al. (2018) and as shown in Fig. S1. A total of 89 rural and 67 urban doormat debris samples were collected (Table S1).

2.3. DNA extraction, amplification and sequencing

Total DNA was extracted from 0.25 ± 0.07 g (target amount ± *SD*) of doormat debris using the PowerSoil® Soil DNA Isolation Kit (QIAGEN GmbH, Germany) following the manufacturer's instructions. Samples were stored at −20 °C until processed further. A highly hypervariable V4 region of the bacterial 16S rRNA gene was

amplified using the primers 515F 5′- GTGCCAGCMGCCGCGGTAA-3′ and 806R 5′-GGACTACHVGGGTWTCTAAT-3′. In the secondary PCR, the full-length P5 adapter and Indexed P7 adapters were used. PCR reactions were performed as described in our previous study (Parajuli et al., 2018); each DNA extraction and MiSeq run had a blank control. The amplicons were sequenced by Illumina MiSeq platform (v3, 2 × 300 bp) in the Integrated Genomic Facility (http://www.k-state.edu/igenomics/) at Kansas State University. Raw sequence reads (paired fastq files) are available in the Sequence Read Archive at NCBI (www.ncbi.nlm.nih.gov) under accession numbers SAMN08991606-SAMN08991884.

2.4. Bioinformatics

Paired-end sequence data (.fastq) from the rRNA gene dataset of the doormat bacterial communities were processed using mothur (version 1.39.5, Schloss et al., 2009) following the protocol by Schloss and Westcott (2011) and Kozich et al. (2013) and as described earlier (Parajuli et al., 2018). Briefly, bacterial sequences were aligned into contigs and any sequences with ambiguous bases, with more than one mismatch to the primers, homopolymers > 8 bp and any without a minimum overlap of 50 bp were removed. The remaining sequences were aligned against a SILVA reference, preclustered to remove erroneous reads (Huse et al., 2010), screened for chimeras with the Vsearch algorithm (Rognes et al., 2016) and non-chimeric sequences were assigned to taxa using the Naive Bayesian Classifier (Wang et al., 2007) against the RDP training set (version 10). Non-target sequences (mitochondria, chloroplast, Archaea) were removed. Sequences were clustered to operational taxonomic units (OTUs) at 97% similarity using nearest-neighbour (single linkage) joining that conservatively assigns sequences to OTUs. Less abundant OTUs that were represented by < 10 sequences across all experimental units were removed to avoid the possibility of PCR or sequencing artefacts (Tedersoo et al., 2010; Brown et al., 2015; Oliver et al., 2015). Bacterial richness and diversity metrics were also measured in mothur. Observed OTU richness (Sobs) and the complement of Shannon diversity and evenness were iteratively calculated and rarefied at 6319 sequences per sample (Fig. S2). For the calculation of diversity indices within each major phylum (and classes under Proteobacteria), the samples were rarefied to adequate sampling depth in each case.

2.5. Garden diversity determination

Plant inventory analysis was performed between June and July in 2015. The number and type of vascular plant species in study participants' yards were recorded using a 0.1-hectare sampling area that excluded roads, forests, fields and buildings. All vascular plant species were classified into the following 10 different morphological-taxonomic categories: shrubs, trees, tree seedlings (1-year-old), non-woody flowering plants (excluding monocots), pteridophytes (ferns), edible berry bushes (e.g. currants), fruit trees (e.g. apple, pear, cherry and plum), non-woody edible plants, perennial plants, and monocots. Owing to a high correlation between some vegetation categories and insufficient number of plant species (particularly in the urban region), the total number of plant species and the number of species belonging to shrubs, trees, non-woody flowering plants and ferns were included in the analyses.

2.6. Statistical analyses

All statistical analyses were performed in the R computing environment (version 3.3.3, R Core Team, 2019). We focused on the effects of sampling month and animal ownership (in the rural area) by conducting separate analyses in rural and urban datasets. This is because the effect of urbanization and particularly the coverage of built area on doormat bacterial communities have been thoroughly addressed in our previous study (Parajuli et al., 2018). Secondly, direct comparison between rural vs urban was prohibited because we included animal owners in the rural dataset but not in the urban dataset. To visualize the bacterial communities, non-metric multidimensional scaling (NMDS) was performed using the vegan package (Oksanen et al., 2015) with Bray-Curtis coefficient as the dissimilarity measure.

The responses of bacterial diversity indices and relative abundances of major taxonomic groups (phyla and classes) to sampling months, animal ownership and vegetation were tested using generalized linear mixed models (GLMM) with the lmer function in the lme4 package in R. The response variables (i.e. diversity indices and percentage relative abundances) were modelled following a Gaussian distribution (Harrison, 2014) and were log or square-root transformed to approximate normality when necessary. Predictor variables included sampling month and animal ownership (rural data only) as a factor and their interaction (rural data only) and five plant inventory variables (number of species of total plants, trees, shrubs, ferns and flowering plants). Since we sampled doormat debris from the same families in February, June and August, doormat samples were added as random terms to the models. We performed model selection by removing non-significant terms, starting with the term with the highest p-value. Plant inventory variables were initially subject to model simplification until only terms with $p < 0.1$ were left. For the rural dataset, if the sampling month by animal ownership interaction remained non-significant ($p > 0.1$) after this procedure, it was also removed. However, to remain true to our experimental design, the main effects, sampling month and animal ownership (rural data only) were always retained in the model irrespective of their significance.

The effect of sampling month, vegetation and animal ownership in rural areas on potential pathogens were tested using GLMM with the glmer functions in the lme4 package in R. We classified bacterial genera using a list of potentially pathogenic species (opportunistic and facultative) published by Taylor et al. (2001) and performed the analysis for the 20 most abundant genera containing pathogenic species. We originally analysed OTUs at species level but present results at genus level. The reasons are that we did not find facultative pathogens, and that opportunistic pathogens contain typically also non-pathogenic strains. In contrast to the percentage relative abundance used for taxa at higher taxonomic level, we used sequence count as the abundance of genera containing opportunistic pathogens because abundant null values in the genus table prevented the data from normality transformation. The count data were modelled following a Poisson error distribution, with an individual-level random effect included to account for possible overdispersion (Harrison, 2014; Hui et al., 2017). However, the predictor variables and model selection strategies were the same in all cases.

The differences in the association between vegetation type and diversity and richness of overall bacterial community and the relative abundance of major phyla between the three sampling time points (June, August and February) were inferred by performing multiple linear regressions separately for June and February in the linear model function using the package MASS (Venables and Ripley, 2013). Regression models were based on both forward and backward elimination of explanatory variables. The model that minimized the Akaike Information Criterion (AIC) value was selected as the final model.

3. Results

3.1. Doormat bacterial community composition

The bacterial 16S rRNA gene sequences from the doormat debris samples were clustered into 15,526 OTUs. This represented 30 bacterial phyla (29 phyla in the rural area and 27 phyla in the urban area). Proteobacteria was the most abundant phylum in both rural and urban samples, accounting for 34% and 39% of the total sequences, respectively. Bacteroidetes was the second most abundant phylum,

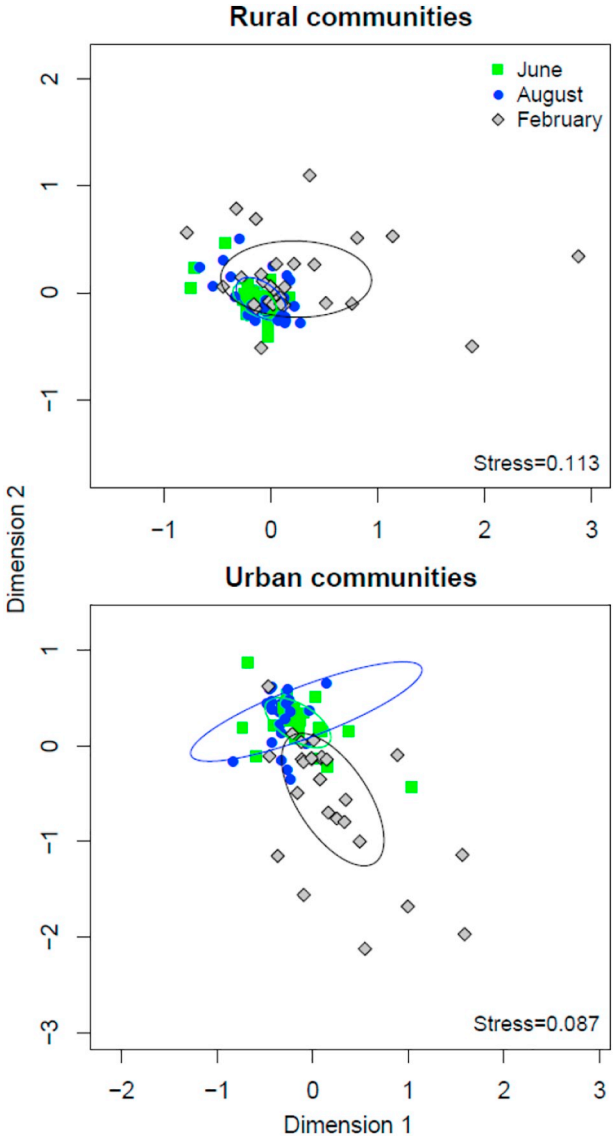

Fig. 1. NMDS ordination reveals a significantly different bacterial community composition across the three sampling months (February, August and June) in rural and urban doormat samples using Bray-Curtis as a dissimilarity metric.

representing 18% (rural) and 13% (urban) of the sequence frequency, followed by Actinobacteria, which represented 18% (rural) and 14% (urban) sequence frequency (Fig. S3).

Permutation analyses (*envfit*) of the bacterial community compositions of winter (February) and summer (June and August) doormat samples indicated that bacterial community compositions differed in the rural ($r^2 = 0.13$, $p < 0.001$) and urban ($r^2 = 0.19$, $p < 0.001$) (Fig. 1) areas, indicating again that doormat bacterial communities in winter did not approximate those in summer. Similarly, the doormat bacterial community compositions also differed between the doormat samples from the houses of animal owners and those without animals ($r^2 = 0.099$, $p < 0.001$; Fig. S4) in the rural doormat samples, suggesting that animals influenced the indoor transfer of environmental microbiota in rural areas.

3.2. Diversity and relative abundance differences due to sampling month and animal ownership

GLMM analyses revealed that the bacterial diversity estimators, namely OTU richness, Shannon diversity and evenness, were the lowest in February among the three sampling months in rural doormat samples (Fig. 2, Table S2). In urban doormat samples, we found a similar trend, specifically bacterial OTU richness and Shannon diversity were greater in summer months (June and August) than in winter (February) (Fig. 2, Table S3). However, the evenness did not differ among the three sampling times (Fig. 2, Table S3), indicating that indoor transfer during summertime did not reduce the proportion of dominant taxa. Bacterial richness had a sampling time and animal ownership interaction (Table S2), plausibly because animal owners had higher doormat richness in June.

We observed a marked reduction in the diversity of major bacterial phyla and classes within Proteobacteria in February doormat samples compared with June and August, particularly in the rural area (Tables S4 and S5 and Fig. S5). In all doormat debris samples, the diversity of Actinobacteria, Acidobacteria, Bacteroidetes, and Proteobacterial classes Alpha-, Beta- and Deltaproteobacteria were consistently lower in February compared with August and June. The diversity of Firmicutes and Gammaproteobacteria was affected by sampling month in rural but not in urban area, and Proteobacterial diversity peaked in August in urban area only (Table S5 and Fig. S5). Interestingly, animal ownership did not affect the diversity of major bacterial phyla and classes except for Firmicutes (increased) and Alphaproteobacteria (decreased) (Table S4 and Fig. S5). In urban samples, the diversity of Proteobacteria was higher in August but not in June compared to February.

The relative abundances of Acidobacteria and Planctomycetes were consistently the lowest in February among the three sampling months (GLMM analysis; Fig. 3, Table S2). In the urban doormat samples, also Bacteroidetes, Chloroflexi, and Verrucomicrobia had the lowest and Firmicutes had the highest relative abundance in February, while these remained constant in rural area among the three sampling months (Fig. 3, Tables S2 and S3). Animal ownership affected the relative

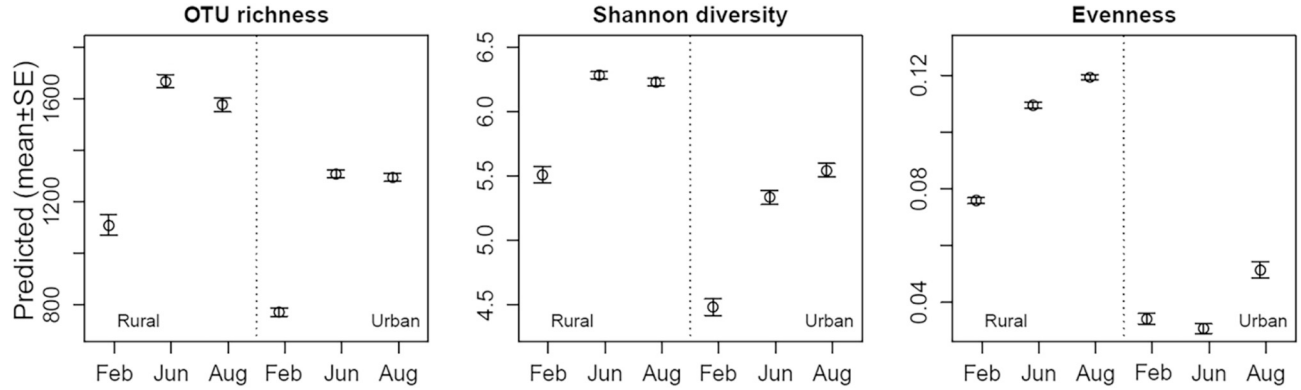

Fig. 2. Differences in OTU richness, Shannon diversity and Evenness among the sampling months in rural and urban doormat samples. Feb = February, Jun = June and Aug = August. For statistics, see Tables S2 and S3. The relative abundances are predicted values from GLMM analysis. The error bars represent standard error.

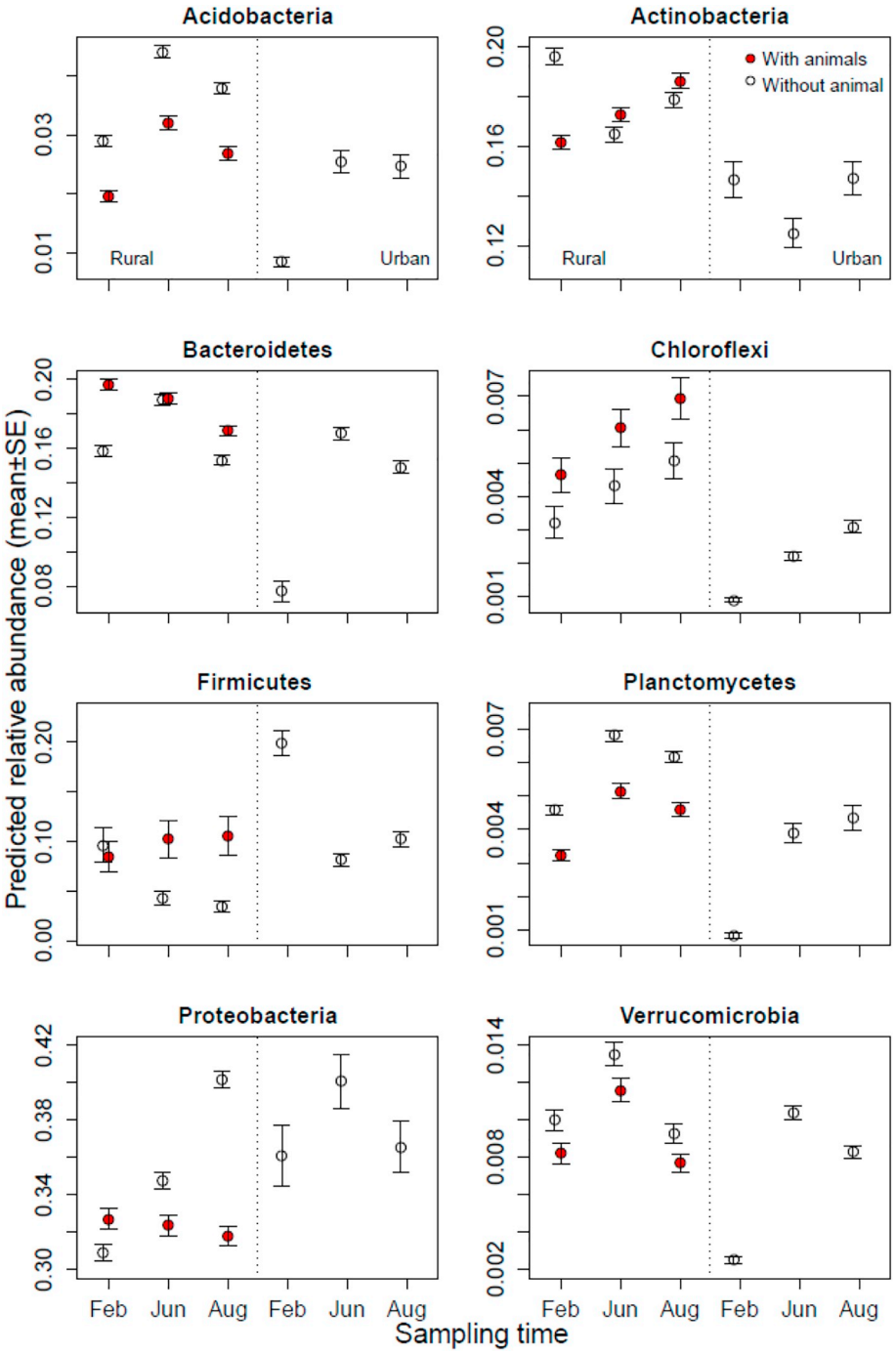

Fig. 3. Temporal differences in the relative abundances of major bacterial phyla in rural and urban doormat samples and the effect of animal ownership in rural doormat bacteria. Feb = February, Jun = June and Aug = August. For statistics, see Tables S2–S3. The relative abundances are predicted values from GLMM analysis. The error bars represent standard error.

abundance of a few bacterial taxa. The relative abundances of Acidobacteria and Actinobacteria were lower, whereas that of Bacteroidetes was higher in doormats from households that owned animals compared to the ones that did not. In addition, sampling time by animal ownership interactions were found in e.g. Proteobacteria and Firmicutes (Fig. 3, Table S2).

At the class level, the relative abundances of Betaproteobacteria, Deltaproteobacteria, Acidobacteria Gp4 and Acidobacteria Gp6 were lowest and that of Bacilli was highest in February among the three sampling months in the rural doormat samples. Animal ownership reduced the relative abundances of Alphaproteobacteria and Acidobacteria Gp1 in the rural doormat samples (Fig. S6). Betaproteobacteria, Acidobacteria Gp4 and Bacilli demonstrated significant sampling time by animal ownership interaction (Table S2). In the urban doormat samples, the relative abundances of Deltaproteobacteria, Acidobacteria Gp1 and Acidobacteria Gp4 were lowest in February among the three sampling times. Bacilli had the highest relative abundance in February among the three sampling months (Fig. S6).

3.3. Association between doormat bacterial community and outdoor vegetation

We observed that the outdoor vegetation had a more profound

association with the doormat microbial communities in winter (February) compared to summer (June) when both rural and urban datasets were analysed together (Tables S6 and S7). In particular, the total number of plant species correlated with the diversity, richness and relative abundance of several bacterial taxa in February, which was less evident in June (Tables S6 and S7). The number of total plant species correlated negatively with the relative abundance of Bacteroidetes ($p = 0.007$) and Gammaproteobacteria ($p = 0.019$) in urban doormats and negatively with Firmicutes ($p = 0.040$) and positively with Proteobacteria ($p = 0.027$) in rural doormats (Tables S2 and S3). Interestingly, the number of plant species was the most important explanatory variable for the variation in the diversity of major bacterial phyla among the plant inventory data in the urban areas. This effect was minimally observed in the rural areas (Tables S4 and S5). When rural and urban datasets were analysed separately and the sampling months were combined in mixed model analyses, none of the plant inventory variables correlated with any bacterial diversity estimators (Table S2 and S3).

3.4. The abundance of genera containing opportunistic pathogens in doormats

We determined the association between the relative abundance of major genera containing opportunistic pathogens and sampling month, animal ownership and plant inventory variables using GLMM analyses in rural and urban areas. Out of the 20 most abundant genera containing opportunistic pathogens, three (*Lactobacillus*, *Staphylococcus* and *Streptococcus*) were enriched in February in both urban and rural doormats. In contrast, only the abundance of *Escherichia* was higher in summer months (June and August) (Fig. 4, Tables S8–S9). Interestingly, animal ownership increased the abundance of *Acinetobacter*, *Bacteroides*, *Chryseobacterium* and *Psychrobacter* in rural areas (Fig. S7 and Table S8). Half of the 20 most abundant genera had sampling month by animal ownership interactions.

4. Discussion

In this study, we aimed to explore the effect of sampling time and outdoor vegetation on the indoor transfer of environmental microbiota. We observed temporal shifts in the bacterial community: diversity and richness of overall bacterial community was reduced and the abundance of taxa comprising of potential pathogens was increased in the winter sample compared to the summer months (Parajuli et al., 2017; Roslund et al., 2018). Our study is the first to provide evidence that seasonal weather conditions are of primary importance in the human exposure to environmental microbiota carried inside in dirt that is attached to shoes and clothing. This is an important revelation for two reasons. First, as direct skin contact to environmental microbiota in dirt is associated with enhanced immunoregulatory responses (Nurminen et al., 2018), our study indicates that these contacts are rare among modern day people who spend a majority of their time indoors (Franklin, 2007). Second, a reduced exposure to a high diversity of environmental microbiota has been suggested to be associated with the increasing incidences of immune-mediated disorders such as allergy and inflammatory disorders (Haahtela et al., 2015).

Another major revelation of our study is that lifestyle factors may partially shape the way weather conditions affect the composition of the microbiota carried inside. In our study, urban dwellers who live in an apartment house had the summertime maximal exposure to environmental microbial diversity at the level of February minimum of rural participants. As richness and diversity of microbiota is generally thought to be crucial in immune modulation and prevention against immune system disorders, our study adds a potential new mechanism for the causes of low exposure to environmental microbiota in urban living environment, i.e. reduced transfer of outdoor microbiota carried inside in the form of litter.

Despite the diminished doormat bacterial diversity, the relative abundances of several bacterial genera comprising of opportunistic pathogens were greater in February than during the summer months. As the relative abundances of Firmicutes genera *Streptococcus*, *Staphylococcus* and *Lactobacillus* were increased in the doormats of both urban and rural areas in February and as the difference among the sampling months was smaller in rural areas, humans seem to be one of

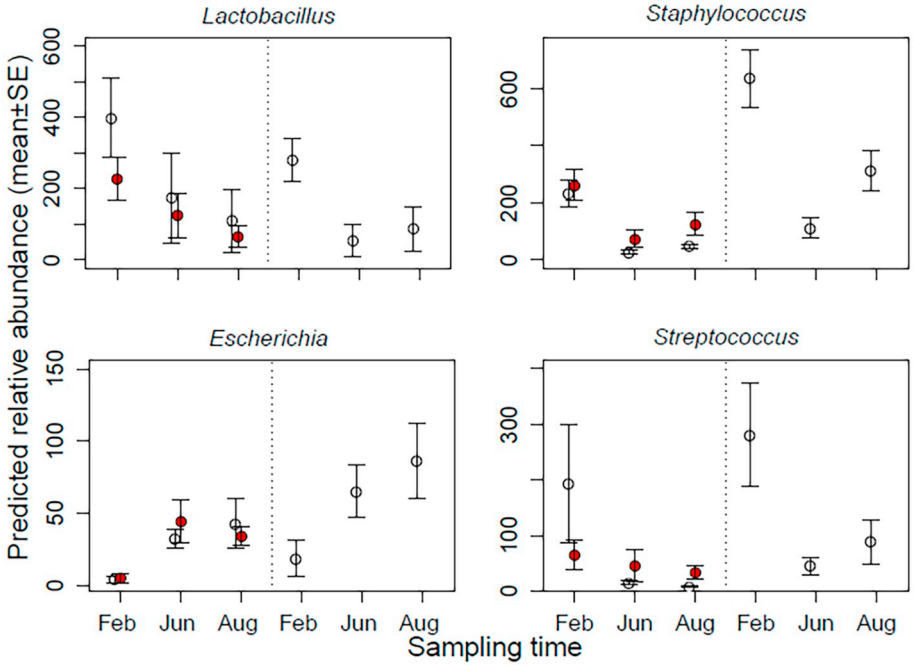

Fig. 4. Temporal differences in the relative abundances of bacterial genera containing opportunistic pathogens in rural and urban doormat samples and the effect of animal ownership in rural doormat bacteria. Feb = February, Jun = June and Aug = August. For statistics, see Tables S8–S9. The relative abundances are predicted values from GLMM analysis. The error bars represent standard error.

the sources of these potentially opportunistic microbes. Despite the general trend of high winter-time abundance of bacterial genera containing pathogenic species, the proportion of *Streptococcus* was lower among animal owners in February (Fig. 4), compared to those participants who did not own a pet or domestic animal. This suggests that pets and domestic animals contribute to *Streptococcus* abundance. Animal ownership altered the relative abundance of *Staphylococcus* as well, particularly when the ground surface was not covered by snow. In contrast, *Escherichia* exhibited reduced abundance in February compared to the summer months in both urban and rural samples. As *Escherichia* are common inhabitants of mammalian and non-mammalian intestines and are also prevalent in the environment (Welch, 2006), *Escherichia* were probably brought indoors through e.g. *Escherichia*-exposed surface soil. The mean relative abundance of these genera (*Streptococcus*, *Staphylococcus*, *Escherichia*) appeared to be higher across all sampling time points in urban doormat litter samples (Fig. 4; see Parajuli et al., 2018). This suggests that people living in urban environments are exposed to a higher abundance of bacterial genera comprising of opportunistic pathogens compared to rural inhabitants. Knowing that exposure to opportunistic pathogens among immunocompromised and chronically ill populations is associated with an increased rate of infections (Groll and Walsh, 2001), our study participants (elderly people) can be expected to be more prone to infections than middle-aged or adolescents. As lifestyle factors contributed to the exposure to opportunistic pathogens in this study, a change in lifestyle and, consequently, reduction in the load of these bacteria could reduce the risk of unwanted infections.

We analysed the effect of garden vegetation on the diversity and relative abundances of individual bacterial taxa detected from the doormats because garden vegetation is a crucial yet underexplored lifestyle factor that is shaped by season. Our analyses led to four interesting findings. Related to the season, doormat bacterial communities responded to garden vegetation more in the winter than in the summer. The plausible explanation is that while soil is under snow, trees, shrubs and many erect perennial plants shed needles, bark and even microscopic plant parts above snow in February (http://puut.luontoportti.fi/index.phtml?lang=en). These then contribute to the bacterial communities carried indoors. This contrasts with summer, when microbiota on soil surface comes into direct contact with shoes and feet and is far more likely to be reflected in the doormat samples. Importantly, the total number of plant species had stronger associations with doormat bacterial communities compared to any specific plant type (see Tables S5–S7). This indicates that microscopic diversity carried inside is related to macroscopic (plant) diversity in the garden. In parallel with the strong associations between doormat bacteria and the total number of plant species, the total number of non-woody flowering plant species correlated with the diversity and richness of the bacterial community (positively) as well as the relative abundance of several bacterial taxa, but only in the summer month (June). No associations between non-woody flowering plants and doormat bacteria were observed in the winter (February). This suggests that the associations may also depend on the state of the plants such as possession of leaves or flowers. The fourth noble finding was that garden vegetation variables correlated with the diversity rather than the relative abundance of major bacterial phyla and classes; the effect was more pronounced in the urban area (Tables S4 and S5). The diversity of Proteobacteria and class Gammaproteobacteria, in particular, were positively associated with the number of non-woody flowering plant species. Our finding fits with the earlier-found association between the diversity of non-woody flowering plants, skin Gammaproteobacterial diversity and atopy (Hanski et al., 2012); we are the first to provide evidence that indoor doormats have a high Gammaproteobacterial diversity if the diversity of non-woody flowering plants in the garden is high.

We also observed that the indoor transfer of several taxa, such as *Acinetobacter* and *Bacteroides*, depended on animal ownership. Studies on human and animal models have shown that Gammaproteobacterial species and particularly *Acinetobacter* and *Bacteroides* (both were enriched in the doormats of animal owners in this study) may have immunomodulatory effects (Round and Mazmanian, 2010; Fyhrquist et al., 2014). However, we studied neither the microbiota of the animals themselves nor took microbiological samples from the outside environment (such as soil, snow and vegetation). We also did not separate pets from domestic animals, simply because several households had both pets (dog, cat) and domestic animals such as cows or horses. Therefore, we cannot precisely state the source of the microbiota that were associated with animal ownership. Nevertheless, the indoor transfer of several taxa depended on animal ownership. As owning animals altered the community composition of doormat microbiota, and as the winter peak in the abundance *Streptococcus* and *Lactobacillus* were lower among animal owners, animals as a lifestyle factor may contribute to the exposure to environmental microbiota and pathogens. Animal ownership should therefore be considered in future studies that aim to explore the effect of environmental diversity on health.

The reduced wintertime diversity and richness of the entire doormat bacterial community and the major bacterial phyla suggest that winter limits indoor transfer of environmental microbiota. The summer peak in diversity was less evident in urban areas where vegetation is limited by asphalt and concrete surfaces, and where most people live in multistorey buildings. An important observation from our study is that the winter minimum in the diversity of rural doormat communities was at the level of the summer maximum in the urban doormat communities (Fig. 2 and Tables S2 and S3). In addition, we observed that the diversity in urban areas in the wintertime was remarkably lower than the minimum observed in rural samples (areas characterized by agricultural activities). This does not only strongly support the hypothesis that biological contamination enriches indoor microbial communities in agricultural areas, but it also indicates that carrying dirt and other debris inside is a major route of microbial transfer; disconnection of man from soil heavily modifies indoor microbial communities. This view is further supported by the differences in evenness; microbial communities in agricultural areas seem to be more diverse, which prevents the prevalence of certain taxa and keeps evenness high. Even richness (the number of species observed per sample) showed the same trend, which is surprising as the samples were subsampled to a sequence number that was available both in rural and urban samples. The fact that we did not observe the same trend uniformly in relative abundances of individual taxa (Figs. 2–4) does not contradict the diversity, evenness and richness findings simply because relative abundances are not actual abundances.

5. Conclusion

Our study indicates that the mimimum (winter month) diversity of environmental microbiota carried indoors in dirt and debris in agricultural areas is higher than the maximum (summer months) diversity in urban areas. This is alarming as a reduced exposure to microbial diversity has been associated with several health disorders (Hanski et al., 2012; Bach, 2002; Ege et al., 2011; Graham-Rowe, 2011; Rook, 2013; Nurminen et al., 2018). Our study also indicates the lack of transfer indoors may be particularly high in areas characterized by a high rate of urbanization and long winters (see Karvonen et al., 2000). Residents of such areas would benefit from prophylactic intervention approaches for increased microbial interaction. This may include different products and services, such as forest materials consisting of high microbial diversity (Puhakka et al., 2018, 2019; Roslund et al., 2018; Grönroos et al., 2018; Nurminen et al., 2018; Hui et al., 2019). Ecologically, we found evidence that both bare soil and macroscopic plant diversity in gardens contribute to the diversity carried indoors in dirt and debris. Whenever the goal is to enhance microbiological diversity indoors, landscaping should allow daily, active and passive contacts with dirt and diverse vegetation in the immediate vicinity of permanent residences.

Based on our findings, we encourage further studies on the impact of indoor-transfer of environmental microbiota on the human immune system. Studies on seasonal effects on indoor microbiota and its correlation with the incidence of these diseases would provide better insight on the association between microbial exposure and immune-mediated disorders. The incidence of certain autoimmune disorders such as childhood type I diabetes mellitus and multiple sclerosis has been reported to be higher among nordic population in general compared to others e.g. southern Europeans and Asians (Karvonen et al., 2000; Koch-Henriksen and Sorensen, 2010). Although this observation is likely due to a multitude of factors, our findings suggest a possible role of reduced microbial exposure and therefore higher prevalence of these diseases in Northern Europe. This observation could be considered in future studies to better understand the effect of climate and geographical variation in the prevalence of immune-mediated non-communicable diseases.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.105069.

Acknowledgements

We acknowledge all the study participants. We thank Kaisa Soikkeli and Jukka Pellinen from AlmaLab (University of Helsinki) for laboratory assistance. We also thank Derek Ho for proof reading the article. Tekes/Business Finland funded the current study (decisions 3347/31/2014 and 6766/31/2017).

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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